Transcriptional activation of the H-ferritin gene in differentiated Caco-2 cells parallels a change in the activity of the nuclear factor Bbf

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In this paper, we examine the mechanisms that regulate the expression of the heavy (H) ferritin subunit in the colon carcinoma Caco-2 cell line allowed to differentiate spontaneously *in vitro*. The differentiation process of these cells in continuous culture is accompanied by an accumulation of the mRNA coding for the apoferritin H chain. The analysis of Caco-2 subclones stably transfected with an H-chain promoter–chloramphenicol acetyltransferase (CAT) construct revealed that the mRNA increase is paralleled by an enhanced transcription of the H gene, driven by the –100 to +4 region of the H promoter. The H gene transcriptional activation seems to be a specific feature of

differentiated Caco-2 cells, since the activity of other promoters did not change upon differentiation. The –100 to +4 region of the H promoter binds a transcription factor called Bbf (B-box binding factor); electrophoretic-mobility-shift-assay analyses showed that the retarded complex due to Bbf–H promoter interaction is significantly increased in the differentiated cells. We propose that the activation of H-ferritin gene expression may be associated with the establishment of a differentiated phenotype in Caco-2 cells, and that the H-ferritin gene transcriptional up-regulation is accompanied by a modification in the activity of the transcription factor Bbf.

INTRODUCTION

Ferritin is an ubiquitous protein whose 24-subunit shell is composed of heavy (H)- and light (L)-subunit types [1]. The relative abundance of these two subunits in the apoferritin shell varies among different cells, and gives rise to L-rich isoforms in liver and spleen, and H-rich isoforms in heart and pancreas [2]. Ferritin plays a central role in intracellular iron metabolism, being involved in the iron intracellular traffic [3] and storage in a soluble form [4]. The iron requirement of many cells varies during their lifetime; accordingly, it has been found that the amount of ferritin and/or its subunit composition are modified during cell proliferation and differentiation [5,6], as well as during neoplastic transformation [7]. The mechanisms of the iron induction of ferritin biosynthesis have been extensively investigated by many groups. Iron regulation takes place essentially in the cytoplasm, where the translation of the H and L mRNAs is controlled by the interaction with a specific translational repressor called IRE-BP (for a review, see [8]).

In addition to the translational control, ferritin gene expression is also controlled at the transcriptional level. Iron itself, when administered for prolonged periods of time, activates ferritin gene transcription [9]. More recently, it has been found that the H-ferritin promoter is activated by thyrotropin [10], geneticin (G418) [11] and insulin-like-growth factor-I (IGF-I) [12], whereas it is down-regulated by the adenovirus E1A protein [13]. Much less is known about the *trans*-acting factor(s) responsible for the modifications of ferritin gene transcriptional activity.

We are interested in understanding the mechanisms by which

the control of ferritin gene transcription is accomplished. The human H-ferritin gene promoter is a 170 bp fragment upstream from the transcription start, with two *cis*-elements recognized by the transcription factors Sp1 and Bbf (B-box-binding factor) respectively [14]. In this work we have analysed the H-promoter activity in Caco-2 cells, a human cell line derived from a relatively well-differentiated colon carcinoma. Caco-2 cells differentiate into enterocyte-like cells as a function of cell density alone, without changes in the culture conditions. Therefore, we used this system to correlate directly H-ferritin gene expression with the cellular differentiated phenotype, thus avoiding any possible interference in the promoter activity of chemical agents added to the culture medium. The results show that during Caco-2 differentiation the H-chain mRNA accumulates, at least in part, as a result of increased transcription. The H-ferritin gene transcriptional activation is driven by the –100/+1 region of the H promoter and is accompanied by increased binding of the nuclear factor Bbf to its *cis*-element.

MATERIALS AND METHODS

Cell culture and enzyme assay

The human colon carcinoma cell line Caco-2 was grown in Dulbecco's modified Eagle's medium (Flow Laboratories), supplemented with 10% (v/v) fetal-calf serum (Hyclone, Logan, UT, U.S.A.), 2 mM glutamine (Flow Laboratories), 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were seeded at a density of about 5×10^5 /10-mm-diam. plastic dish and routinely

Abbreviations used: H, heavy; L, light; Bbf, B-box-binding factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase; DAO, diamine oxidase; EMSA, electrophoretic mobility shift assay.

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subcultured when about 80 % confluent, which was around day 6. Diamine oxidase (DAO) activity and sucrase activity were assayed as described previously [15].

Northern blot analysis

Total RNA was extracted from cells at different days of culture with the phenol/chloroform method described by Chomczynsky and Sacchi [16]. Samples (5 µg) of total RNA were size-fractionated on a 2.2 M formaldehyde/1.5 % agarose gel, transferred to a nitrocellulose filter (Nytran; Schleicher and Schuell, Dassel, Germany) and probed as described previously [11]. The ³²P-labelled probes were: a 528-bp *Pst*I fragment of the H-ferritin cDNA [17]; a 1600-bp *Bam*HI fragment from the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [18]; and a 1200-bp *Hind*III-*Bam*HI fragment from the Rous sarcoma virus (RSV)-hygro plasmid [19].

Isolation of stable transfectants and chloramphenicol acetyltransferase (CAT) assays

For the isolation of stable transfectants, the ferritin 5' HH/A construct [14] and the RSV-hygro plasmid [19] or the pSV2-CAT [20] and the RSV-hygro plasmids were introduced into Caco-2 cells by the calcium phosphate precipitation method [21]. After 2 days the cells were trypsinized and transferred into 12-well plates, where selection was carried out in the presence of 800 µg/ml hygromycin B (Sigma). Pools of clones were picked up after about 3 weeks. CAT assays were performed as described previously [11].

Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were prepared from Caco-2 cells as described previously [11]. The B-element, Sp1 and CTF/NF1 probes used in the EMSA were prepared by labelling 1 pmol of double-stranded oligonucleotides with 16 units of T4 polynucleotide kinase (Promega, Madison, WI, U.S.A.) and about 40 µCi of [γ -³²P]ATP (Amersham) for 30 min at 37 °C. The labelled oligonucleotides were then purified from non-denaturing 10 % polyacrylamide gels. About 4 × 10⁴ c.p.m. of eluted probe was used for EMSA. Alternatively, the region of the H promoter from position -100 to +4 was terminally labelled and 0.5 ng of DNA used for EMSAs. EMSAs were performed as described previously [11]. The oligonucleotides used were: B, 5'-CGGCGCTGATT-GGCCGGGGCGGGC-3'; CTF/NF1, 5'-TTATTTTGGATT-GAAGCCAATATGATAA-3'; and Sp1, 5'-GGGCGGGGGC-GGGGGCGGGGGCGG-3'. In the competition assays, the nuclear extracts from 3-day-old and from 14-day-old cells were preincubated for 20 min with increasing concentrations of unlabelled specific competitor (-100 to +4 region) as indicated; then 0.5 ng of ³²P-radiolabelled probe (-100 to +4 region) was added to the reaction mixtures. After 30 min incubation the reaction products were analysed by EMSA as described [11]. Alternatively, nuclear extracts were preincubated for 20 min with a 100 molar excess of unlabelled -100/+4 DNA for the indicated times prior to the addition of the labelled probe. After 30 min incubation the reaction products were analysed by EMSA.

RESULTS

Transcription of the H-ferritin gene during Caco-2 differentiation

The transcriptional and post-transcriptional mechanisms controlling ferritin gene expression control have been studied in

Figure 1 H-ferritin mRNA levels and DAO activity in Caco-2 cells at different days of culture

Northern blot analysis was performed as described in the Materials and methods section. Total RNA from Caco-2 cells harvested at different days of culture were analysed by (a) hybridization with a radiolabelled H-ferritin cDNA and with a GAPDH probe as control of RNA loading. (b) DAO activity in the culture medium from Caco-2 cells at different days.

many experimental systems [5,11,12,22]. We explored the functions of the H-ferritin gene in the Caco-2 human cells, which differentiate spontaneously into enterocyte-like cells with time, under standard culture conditions. Caco-2 differentiation starts upon confluency, and is accompanied by morphological modifications and by the expression of some enzymes peculiar to the adult small intestine [23]. To follow the differentiation process under our culture conditions, we measured the DAO activity, a differentiation marker, in the medium of Caco-2 cells on different days. The DAO activity in the medium started to increase around day 4 and reached a plateau at day 12 (Figure 1b). In addition, DAO activity and sucrase activity were analysed in the cell lysates; the two enzymic activities also started to rise around day 4, and reached a maximum at 14 days (results not shown).

The amount of ferritin H-chain mRNA present during this *in vitro* differentiation process was analysed by Northern blot analysis of the total RNA extracted from the cells harvested on different days. As shown in Figure 1(a), the steady-state amounts of the H-chain mRNA increased about 5-fold around day 6 and remained unchanged up to day 14.

Both transcriptional efficiency and half-life of the transcripts are potential sites of regulation of ferritin gene expression [9–13,22]. To evaluate whether the increase of H-ferritin mRNA found in differentiated Caco-2 cells reflects an enhanced transcription efficiency of the H gene promoter, we generated Caco-2 stably transformed subclones containing the bacterial CAT gene under the control of the -100 to +4 region of the H promoter (5' HHA construct [14]). Pools of transfectants were isolated by selection with 800 µg/ml hygromycin B for 2 weeks, and then analysed for the presence of the 5' HHA construct. Two

Figure 2 H-ferritin and SV40 promoter activities in Caco-2 stable transfectants

(a) CAT activity driven by the H-ferritin promoter in Caco-2 stable transfectants harvested at different days. The values are the means of two independent experiments performed on two different pools and are expressed as percentages of the acetylated form of the [^{14}C]chloroamphenicol. (b) CAT activity driven by the SV40 early promoter in Caco-2 stable transfectants. The values are the means of two independent experiments performed on two different pools and are expressed as percentages of the acetylated form of the [^{14}C]chloroamphenicol. (c) Northern blot analysis of Caco-2 stable transfectants; total RNA was extracted from the cells at different days and probed with a hygromycin probe, as described in the Materials and methods section.

independently obtained pools of subclones were allowed to differentiate in culture for 14 days, and the transcription of the CAT gene, driven by the human H promoter, was determined on different days. Figure 2(a) shows the results obtained with the above-mentioned transfectants. The CAT activity in the extracts from 14-day-old differentiated cells is about 7-fold higher than in the extracts from 2-day-old undifferentiated cells. Most of this increase takes place around day 6. The levels of H-ferritin mRNA in these transfectants, measured by Northern blot, show the same profile of increase (results not shown).

The enhanced transcription driven by the H promoter did not reflect a generalized increase of transcription in Caco-2 transfectants upon differentiation. With the same procedure described above, we also generated Caco-2 stable transformants in which the transcription of the CAT gene was driven by the simian virus 40 (SV40) early promoter [20]. In two independently obtained pools of transfectants, cultured for 14 days, the CAT activity did not change following differentiation (Figure 2b). In addition, the transcription of the hygromycin mRNA, driven by the RSV

Figure 3 EMSA of the B element of the H-ferritin promoter

(a) Samples (4 μg) of nuclear extracts from Caco-2 cells at 3, 7, 10 and 14 days were challenged with the region of the H promoter from -100 to +4, terminally labelled. Key to lanes: free probe, no nuclear extract; Comp. sp., the nuclear extract from 14-day-old cells was preincubated with a 100-fold molar excess of unlabelled -100 to +4 DNA fragment as specific competitor; Comp. asp., the nuclear extract from 14-day-old cells was preincubated with a 100-fold molar excess of aspecific competitor oligonucleotide. (b) Samples (4 μg) of nuclear extracts from Caco-2 cells at 7 and 14 days were challenged with a B-site oligonucleotide. Free probe: no nuclear extract. Comp.: the nuclear extracts from 7 and 14 days cells were preincubated with a 100-fold molar excess of unlabelled oligonucleotide. (c) Samples (4 μg) of nuclear extracts from Caco-2 cells at 7 days were challenged with the -100/+4 probe terminally labelled. Free probe: no nuclear extract. Comp. sp.: the nuclear extract was preincubated with a 100-fold molar excess of unlabelled -100 to +4 DNA fragment. Comp. asp.: the nuclear extract was preincubated with a 100-fold molar excess of aspecific competitor oligonucleotide. Sp1: the nuclear extract was preincubated with a 100-fold molar excess of unlabelled Sp1-site oligo. (d) Samples (4 μg) of nuclear extracts from Caco-2 cells at 3, 7, 10 and 14 days were challenged with an Sp1 oligonucleotide and with a CTF/NF1 oligonucleotide. Free probe: no nuclear extract. Comp.: nuclear extracts were preincubated with a 100-fold molar excess of unlabelled Sp1 and CTF/NF1 oligonucleotides, respectively.

promoter [19], did not change during Caco-2 differentiation, as measured by Northern blot analysis (Figure 2c).

Bbf of the H-ferritin promoter in Caco-2 cells

The analysis of Caco-2 stable transfectants demonstrates that the

Figure 4 Competition assay with 3-day-old and 15-day-old Caco-2 nuclear extracts

Samples (4 µg) of nuclear extracts from Caco-2 cells at 3 and 14 days were pre-incubated for 20 min with the indicated molar excess of unlabelled -100 to +4 region prior to the addition of the ³²P-labelled probe (-100/+4 region). After 30 min incubation samples were analysed by EMSA. Free probe: no nuclear extract.

-100 to +4 region of the H promoter is responsible for the transcriptional activation during cell differentiation. The -100 to +4 region of the H promoter contains the B *cis*-element, whose sequence is highly conserved in humans and rat, and which is recognized by a ubiquitous transcription factor called Bbf [14]. Thus we sought to compare DNA-protein interactions of the B element in differentiated and undifferentiated Caco-2 cells. Nuclear extracts from cells harvested at different days were challenged with the -100 to +4 region of the H promoter, and analysed by EMSA. As shown in Figure 3(a), the intensity of the specifically retarded complex increased linearly with time of culture. To confirm that this complex did in fact contain the Bbf, a similar experiment was performed using a synthetic double-stranded B-site oligonucleotide. As shown in Figure 3(b), the result obtained with the B-site oligonucleotide is identical to that obtained with the -100/+4 probe. In addition, competition assays with an Sp1-site oligonucleotide did not affect the formation of the B-site/Bbf complex (Figure 3c). The results of the EMSA indicate that the H-ferritin gene transactivation in differentiated cells is accompanied by a rather specific accumulation of Bbf and/or an increase in its DNA-binding activity, because the levels of two other transcription factors, namely Sp1 and CTF/NF1, did not change upon differentiation (Figure 3d).

We next analysed the Bbf-H promoter complex in undifferentiated and differentiated cells by competition assays. In an initial approach, we challenged the nuclear extracts with increasing amounts of unlabelled B-site DNA, prior to the addition of the radiolabelled B-site probe. As shown in Figure 4, Bbf from differentiated Caco-2 cells (day 15) was almost completely saturated by pre-incubation with a 10-fold excess of unlabelled competitor, whereas the same 10-fold excess had no effect on the signal obtained with extracts from undifferentiated cells (day 3). In a second approach, the nuclear extracts were pre-

incubated with a 100-fold molar excess of unlabelled probe for different times prior to the addition of the ³²P-labelled fragment. EMSA was then used to visualize the fraction of Bbf able to form the complex after the pre-incubation with the unlabelled competitor. The results are shown in Figure 5(a). In the extracts from differentiated cells (14 days), a 10 min pre-incubation with the unlabelled competitor is sufficient to compete fully for the formation of the Bbf-radiolabelled B-site complex. The competition experiments suggest that Bbf saturation with the unlabelled probe occurs at a lower DNA concentration and more rapidly with the extracts from differentiated cells than with those of undifferentiated cells. The experiment shown in Figure 5(c) was performed as described above, except that the amount of nuclear proteins from 14-day-old Caco-2 cells was normalized to that of the 2-day-old cell extracts. We also analysed Sp1 with the same approach in extracts from 14- and 2-day-old cells (Figure 5d) and found that binding of Sp1 remains almost unchanged in the two types of nuclear extracts.

DISCUSSION

A fully differentiated cell is the end-product of the expression of a programmed repertoire of genes. A number of studies, performed on different cell types, demonstrated that the expression of ferritin genes is differentiation-regulated. The level of H- and L-chain mRNAs is altered during *in vitro* differentiation of HL60 cells [5], of Friend erythroleukaemic cells [6], and of cultured human blood monocytes [24]. The role of ferritin genes in differentiation is not restricted to the haematopoietic compartment; changes in the amount of transcripts have also been found in rat thyroid cell differentiation [10] and in rabbit vascular-smooth-muscle cells [25]. In most of these cases the differentiation is obtained upon addition of chemical substances to the culture medium, and it has been shown that the H-ferritin gene is responsive to different exogenous stimuli. Our results on the spontaneous process of enterocyte-like differentiation of Caco-2 cells increases the number of cell types in which the amount of H ferritin mRNA is modulated as a function of the differentiation status, and confirms a direct relationship between the acquisition of a differentiated phenotype and modifications of ferritin gene expression. In Caco-2 cells, the enhanced expression of the H-ferritin gene seems to be a rather early phenomenon, since the amount of the mRNA increases at day 6, when the differentiation process of Caco-2 cells is still not fully accomplished.

The analysis we performed on Caco-2 stable transformants indicates that the accumulation of the H-chain mRNA in Caco-2 differentiated cells is, at least in part, due to the transcriptional activation of the gene. The DNA *cis*-elements involved in this activation are restricted to the 100 nucleotides upstream from the transcription start point. We demonstrated that the retarded complex resulting from the binding of Bbf to the -62 to -45 region of the H promoter increases during Caco-2 differentiation, thus paralleling the increased transcriptional efficiency of the H-chain gene. Sp1, which binds the H promoter upstream from the -100 region (position -132 to -109), remains almost unchanged in amount and/or affinity for the DNA. Competition experiments suggest, in differentiated cells, a greater efficiency in the formation of the Bbf-DNA complex compared with the undifferentiated cells. In a previous study we demonstrated that Bbf plays a key role in the H-chain transcriptional increase observed in cells chronically treated with G418 [11]. The findings obtained with Bbf in differentiated Caco-2 cells confirm the functional role of this nuclear factor in the transcriptional control of the H-ferritin gene.

Figure 5 Competition assay with 2- and 14-day-old Caco-2 nuclear extracts

(a) Samples (4 μg) of nuclear extracts from 2- (2d) and 14-day-old (14d) Caco-2 cells were incubated with a 100-fold molar excess of unlabelled — 100 to +4 fragment for 0, 10, 15 and 30 min prior to the addition of the ^{32}P -labelled fragment. After 30 min incubation the samples were analysed by EMSA. (b) The results of the experiment shown in (a) were quantified by densitometric scanning of the film and expressed as percentage of ^{32}P -labelled fragment competed for by unlabelled DNA. Closed circles refer to extracts of 14-day-old cells and open circles to the extracts of 2-day-old cells. (c) The experiment was performed as the one shown in (a), by using 4 μg of nuclear extracts from 2-day-old and 1 μg of nuclear extracts from 14-day-old cells. (d) The experiment was performed as the one in (a) with 4 μg of nuclear extracts from 2-day-old and 14-day-old Caco-2 cells challenged with an Sp1-site oligonucleotide.

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